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used to discriminate among trees and showed that Tree 1 was 0.598 FREQPARS units shorter than Tree 2. There was both congruence and complementarity in the support provided by allozymic and morphological datasets. The sister-group relationship and allopatric distributions of *E. beanii* and *E. bifascia* are consistent with their origin resulting from vicariant speciation associated with the origin of the Mobile Basin.

Key words: Etheostoma, Ammocrypta, allozymes, historical biogeography, phylogeny

INTRODUCTION

The sand darters are slender, elongate, translucent darters known for their habit of burying themselves in sandy substrates. Six species of sand darters are currently classified in the subgenus *Ammocrypta*: *Etheostoma pellucidum, E. beanii, E. vivax, E. clarum, E. bifascia*, and *E. meridianum*.

The taxonomic history of *Ammocrypta* began with its use as a generic name by Jordan (1877) with his description of *A. beanii*. Three additional species now included in the subgenus *Ammocrypta* (originally described as *Pleurolepis pellucida, A. vivax*, and *A. clara*) were described by 1886 (Agassiz, in Putnam, 1863; Hay, 1883; and Jordan and Meek, 1885, respectively). Bailey and Gosline (1955) added *Crystallaria asprella* to the genus *Ammocrypta*, but placed it in a monotypic subgenus *Crystallaria*. Although Bailey and Gosline (1955) argued for a classification of darters containing three genera (*Percina, Ammocrypta*, and *Etheostoma*) that was generally accepted, a few authors (e.g., Moore, 1968; Miller and Robison, 1973) continued to recognize *Crystallaria* as a monotypic genus. Williams (1975) described two additional species within the subgenus *Ammocrypta*. Within this subgenus, his *A. beanii* group consisted of the species with a few scale rows (*E. beanii, E. bifascia*, and *E. clarum*) and his *A. pellucida* group contained the partially to almost completely scaled species (*E. pellucidum, E. meridianum*, and *E. vivax*).

Williams (1975) considered the genus *Ammocrypta* more closely allied to the subgenus *Imostoma* of the genus *Percina* than to *Etheostoma*. However, Page and Whitt (1973a) argued that *Percina* was monophyletic and suggested that *Ammocrypta* was related to *Etheostoma*; this conclusion was based on a unique $LDHB_4$ isozyme found only in *Percina*. (Their assessment of the polarity of this character is unclear—in their Fig. 4, p. 6, *Etheostoma* and *Ammocrypta* are united by a derived LDH B_4 isozyme.) Page and Whitt (1973b) stated that the TO isozyme shared by *E.* (*Vaillantia*) *chlorosoma* and *A. pellucida* indicated that *Ammocrypta* was more closely related to *Etheostoma* than to *Percina*.

Simons (1989, 1991) removed *Crystallaria* from *Ammocrypta* and hypothesized that the phylogenetic position of *Crystallaria* was basal to *Etheostoma* and *Percina*. Simons (1989, 1992) subsumed the remaining six species of *Ammocrypta* within the genus *Etheostoma* and suggested that *Etheostoma* (*Ioa*) *vitreum* was the sister group to the subgenus *Ammocrypta*. This change resulted in four subgenera being included in the *Boleosoma* group of *Etheostoma*: *Boleosoma, Ioa, Vaillantia*, and *Ammocrypta*. Simons' hypothesis of relationships within the subgenus *Ammocrypta* differed somewhat from those postulated by Williams (1975). Simons (1992) did find support for the *E. pellucidum* group; *E. meridianum* and *E. pellucidum* were sister taxa, and *E. vivax* was the sister group to this pair. However, Simons (1992) did not find support for the *E. beanii* group; instead, the species pair *E. beanii-E. bifascia* was the sister group to the *E. pellucidum* group and *E. clarum* was the sister group to all other members of the subgenus *Ammocrypta*.

The relationships hypothesized by Simons (1989, 1991, 1992) and based on phylogenetic analyses, have not been widely accepted. For example, Etnier and Starnes (1993), Jenkins and Burkhead (1994), Mettee et al. (1996), and Pflieger (1997) all continued to recognize the genus *Ammocrypta* in their state guides (Tennessee, Virginia, Alabama, and Missouri, respectively). Although Jenkins and Burkhead (1993) alone justified their retention of the genus *Ammocrypta*, in no case is an explicit alternative phylogenetic analysis or hypothesis presented. Wood and Mayden (1997) however, found support for a sister-group relationship between *Etheostoma beanii* and *Crystallaria* in maximum parsimony analyses and in their most parsimonious FREQPARS tree, but they did not make any taxonomic recommendations based on this result. In the absence of an explicit alternative phylogenetic analysis of more than two taxa, we continue to consider members of *Ammocrypta*, members of the genus *Etheostoma*, subgenus *Ammocrypta*.

The particular relationships of these species are of interest to biogeographers. Wiley and Mayden (1985) followed the relationships suggested by Williams (1975) and postulated that the distribution of members of the *Etheostoma beanii* group was the result of a western vicariance event loosely associated with the Mississippi River. *Etheostoma clarum* is found in the Mississippi River Basin and drainages to the west, and the presumed sister taxon (*E. beanii-E. bifascia*) is found east of the Mississippi River mainstem. Wiley and Mayden (1985) also suggested that the distributions of two other groups could be attrib-

uted to vicariance events involving the Mobile Bay Basin. *Etheostoma beanii* is found in the Mobile Basin and westward to the Mississippi River mainstem, whereas *E. bifascia* is only found east of Mobile Bay. A similar situation is found in the *E. pellucidum* group. *Etheostoma meridianum* is a Mobile Basin endemic, whereas *E. pellucidum* and *E. vivax* are found in drainages north and west of the Mobile Basin. Simons' (1992) results did not substantiate the vicariance event associated with the Mississippi River or a vicariance event between *E. meridianum* and the species pair *E. vivax-E. pellucidum*.

The main purpose of this study is to reexamine the relationships of members within the subgenus *Ammocrypta* and the controversial relationship of the subgenera *Ammocrypta* and *Ioa* by including new data from electrophoretic studies. A secondary purpose of this study is to reexamine the biogeographic history of the subgenus *Ammocrypta*.

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MATERIALS AND METHODS

Taxa examined.—All six members of the subgenus *Ammocrypta* were included in the ingroup. Outgroups were chosen using the hypothesis of Simons (1992) as a guide. *Etheostoma nigrum* and *E. longimanum* were included as representatives of subgenus *Boleosoma*. These species have the potential to act as the first (close) taxonomic outgroup. Within *Boleosoma*, his clade consisting of *E. nigrum*, *E. olmstedi*, and *E. perlongum* is represented here by *E. nigrum*. The species pair *E. longimanum-E. podostemone* is represented by *E. longimanum*. Although *E. nigrum* and *E. longimanum* were included in the analysis as taxa outside the taxonomic group of interest (*Ammocrypta* and *E. vitreum*), they were not designated as outgroups in the analyses. The second (distant) taxonomic outgroup was *E. davisoni*. This species represents the species pair that make up the subgenus *Vaillantia* and was designated as the sole outgroup in the analyses.

Enzyme electrophoresis.—Fishes were collected by seining, frozen immediately in liquid nitrogen, transported to the laboratory, and stored at –70° C for up to 5 yr (see Appendix 1—Specimens Examined). Skeletal muscle, liver, and brain/eye tissues were dissected and homogenized separately in a 1:1 (v:v) mixture of tissue and 0.01 M Tris, 0.001 M EDTA, and 0.001 M mercaptoethanol, pH 6.8. Homogenates were centrifuged at 15,000x *g* for 10 min at 5° C. Within 72 h, the supernatant fractions were electrophoresed at 5° C on horizontal starch gels composed of 12% hydrolyzed potato starch (Starch Art Corp.). Histochemical staining protocols did not differ substantially from those of Murphy et al. (1990).

Enzyme nomenclature follows the recommendations of the International Union of Biochemistry Nomenclature Committee (1984), and locus nomenclature follows the recommendations of Buth (1983). Enzymes, loci, tissue sources, and electrophoretic conditions are listed in Appendix 2. Twenty-seven presumptive gene loci were visualized by histochemical staining. Electromorphs for each locus were coded a, b, c, etc., in order of increasing anodal mobility. These designations are relevant to this study only. Two loci were fixed (M-lcdh-A and Ldh-A) for all taxa sampled and not included in the analyses. Two loci were variable only within single species (Ldh-B within *Etheostoma meridianum* and Tpi-B within *E. vivax*), and these autapomorphies were not considered further. The remaining 23 variable loci were considered independent transformation series (TS), and each allele was considered a unique state (TS 1–TS 23, Appendix 3).

Morphological characters.—Twenty-nine morphological transformation series originally described by Simons (1992) were included in this study. Many of the descriptions of these transformation series have been slightly revised, and for this reason they are presented below. Transformation series and state numbers refer to those presented in Appendix 3 (TS 24–TS 52).

TS 24.—Ascending process of the premaxilla: (0) perpendicular to the alveolar process or (1) reclined posteriorly.

TS 25.—Maxillary process of the premaxilla: (0) not elongate or (1) elongate and enlarged.

TS 26.—Premaxillary socket of the maxilla: (0) V-shaped, with the lateral and medial walls approximately equal in length or (1) U-shaped, with the lateral wall curving medially and longer than the medial wall.

TS 27.—Palatine teeth: (0) present or (1) absent.

TS 28.—Notch posteroventral to the articular process of the quadrate: (0) shallow to absent or (1) cut deeply into the body of the quadrate.

TS 29.—Body of the quadrate: (0) rounded, with a

notch between the body and the posterior process of the quadrate or (1) rectangular, without a notch.

TS 30.—Hyomandibular struts: (0) present as cruciform thickenings within the hyomandibula or (1) extremely reduced to absent.

TS 31.—Descending process of the hyomandibula: (0) long, extending beyond the preopercular groove or (1) short, terminating at the end of the preopercular groove.

TS 32.—Hyomandibular spur: (0) absent or (1) present.

TS 33.—Ventral plate of the urohyal: (0) flattened, with the insertion for the urohyal–hypohyal ligaments directed anteriorly from the anterior surface or (1) curved, with the insertion sites directed anteroventrally from the ventral surface. Simons (1992) considered this character homoplastic, because in his analysis it supported the monophyly of the subgenera *Boleosoma* and *Ioa*.

TS 34.—Articular process for the interhyal on the posterior ceratohyal: (0) present or (1) absent.

TS 35.—Posterior margin of the preopercle: (0) smooth or (1) serrate with a few points projecting beyond the margin of the bone.

TS 36.—Notch in the anterior angle of the preopercle: (0) present, roofing the articulation for the interhyal or (1) absent.

TS 37.—Opercular spine: (0) present or (1) absent.

TS 38.—Opercular strut: (0) strong, extending from the hyomandibular articulation to or almost to the posterior margin of the opercle or (1) greatly reduced, extending less than half the distance to the margin.

TS 39.—Posterodorsal extension of the subopercle: (0) elongate and filamentous or (1) truncated near the dorsal margin of the opercle.

TS 40.—Mesethmoid: (0) thick and expanded anteriorly, extending anteriorly beyond the lateral ethmoids or (1) thin and concave anteriorly, not extending beyond lateral ethmoids. This character was considered homoplastic by Simons (1992), because in his analysis it supported grouping together the subgenera *Vaillantia, Boleosoma*, and *Ioa*.

TS 41.—Maxillary ligament insertion: (0) on two dorsolateral projections of the mesethmoid, (1) on a single dorsomedian knob of the mesethmoid, or (2) on two dorsomedian ridges of the mesethmoid. Simons (1992) considered this character homoplastic because in his analysis it supported grouping together the subgenera *Boleosoma* and *Ioa*.

TS 42.—Vomerine teeth: (0) always present or (1) usually absent.

TS 43.—Membrane bone on the lateral margin of the nasal: (0) extensive, overlying the olfactory capsule or (1) reduced to a thin slip along the canal.

TS 44.—Remnant of the lateral line canal of the supracleithrum: (0) present or (1) absent.

TS 45.—Postcleithrum 2: (0) present or (1) absent.

TS 46.—Longitudinal struts on the anal proximal pterygiophores: (0) present or (1) absent.

TS 47.—Process for the insertion of the *m. infracarinalis medius* on the anterior face of the first anal pterygiophore: (0) present or (1) absent. This character was considered homoplastic by Simons (1992), because in his analysis it supported a group consisting of *Etheostoma beanii, E. chlorosoma, E. davisoni, E. stigmaeum*, and *E. jessiae*.

TS 48.—Swollen, thickened tips of fin spines and rays of breeding males: (0) absent or (1) present, such that the tip of the pelvic spine is covered by a large fleshy knob and the ventralmost pectoral rays and pelvic rays are swollen and thickened at the tips.

TS 49.—Body squamation: (0) almost complete or (1) reduced laterally to a few rows of scales.

TS 50.—Tubercles on pelvic fins of breeding males: (0) present or (1) absent. This transformation series and the next one were both part of a single transformation series in Simons' (1992) analysis. Although tuberculation is usually considered a single multistate variable, the presence of tubercles on many areas of the body occur independently across all taxa of darters and are considered different transformation series in this study. That is, the presence of tubercles on the pelvic fins does not show a one-to-one correspondence with the presence of tubercles on the anal fin. Tubercles on the pelvic fins of breeding males have been observed for many darters, including: *Percina evides, P. palmaris, P. shumardi, P. vigil, P. aurantiaca, P. copelandi, Crystallaria asprella* and, within the genus *Etheostoma*, all members of the subgenera *Allohistium, Ammocrypta, Doration*, and *Ioa*, and *E. chlorosoma, E. punctulatum, E. boschungi, E. cragini, E. pallididorsum, E. australe, E. hopkinsi, E. spectabile, E. luteovinctum, E. serrifer, E. gracile, E. zonifer, E. fusiforme, E. saludae, E. collis, E. proeliare, E. microperca*, and *E. fonticola* (Collette, 1965; Bailey and Etnier, 1988; Jenkins, 1971; and S. R. Layman, pers. comm.).

TS 51.—Tubercles on the anal fins of breeding males: (0) absent or (1) present (Collette, 1965; Bailey and Etnier, 1988; Jenkins, 1971; and S. R. Layman, pers. comm.). Anal fin tubercles have been observed in *Etheostoma parvipinne, E. fricksium, E. radiosum, E. whipplei*, and *E. trisella*; these five taxa lack pelvic fin tubercles. Of the taxa that exhibit pelvic fin tubercles (listed above), only *E. pellucidum, E. meridianum*, and *E. vitreum* lack anal fin tubercles. All members of the subgenera *Etheostoma, Ulocentra* (*sensu* Bailey and Etnier, 1988), and *Boleosoma* lack anal (and pelvic) fin tubercles, as does *E. davisoni*.

TS 52.—Shape of the female genital papilla: (0) conical, (1) cupped, or (2) flat and bilobed. Variation in the shape of the female genital papilla is widespread among members of *Etheostoma*; however, most species have short,

conical papillae. Of the taxa included in this analysis, female *E. davisoni* have cup-like papillae (Howell, 1968), female *E. longimanum* and *E. nigrum* have bilobed, flattened papillae (Cole, 1967), and all females of the subgenera *Ioa* and *Ammocrypta* have conical papillae.

Some of the transformation series used by Simons (1992) are not included here, because they are uninformative in this study. They were either synapomorphic for the entire *Boleosoma* group (his characters 3 and 4), provided support for groups within the subgenus *Boleosoma* (his characters 7, 24, 25, and 32), supported the subgenus *Vaillantia* (his characters 10, 35, 36, and 38 [in part]), or were autapomorphic for *Etheostoma vitreum* (his characters 29 and 37 [in part]).

Phylogenetic analyses.—The underlying methodology for this study is phylogenetic parsimony analysis (Hennig, 1966; Kluge and Farris, 1969; Wiley, 1981; Farris, 1983; Churchill et al., 1985; Farris and Kluge, 1985, 1986). The precept that all available evidence must be brought to bear on any statement about relationships (Kluge, 1989) is also adhered to. The outgroup comparison method has been shown to be the most comprehensive for polarizing hypotheses of transformation (Stevens, 1980; Farris, 1982; Kluge, 1984, 1985; Brooks and Wiley, 1985), and consequently, it was used in this study.

A variety of analyses were performed on the allozyme data alone and on the allozyme data in conjunction with the morphological data: (1) analyses of allozyme data using BIOSYS-1 (Swofford and Selander, 1981), (2) qualitative analyses of allozyme and combined allozyme and morphological data using PAUP 3.1.1 (Swofford, 1993), and (3) quantitative analyses of the topologies produced from the preceding BIOSYS-1 and PAUP 3.1.1 analyses utilizing FREQPARS (Swofford and Berlocher, 1987; Swofford, 1988).

For BIOSYS-1 analyses, genotype frequencies from the allozyme data were used to construct topologies following a variety of methods: Rogers (1972) genetic distance, modified Rogers distance (Wright, 1978), Prevosti distance (Wright, 1978), Cavalli-Sforza and Edwards (1967) chord distance, Cavalli-Sforza and Edwards (1967) arc distance, and Edwards (1971, 1974) "E" distance. These genetic distances were summarized in distance-Wagner trees (Farris, 1972) using Swofford's (1981) Multiple Addition Criterion Procedure and were rooted with the single outgroup, *Etheostoma davisoni*. The resultant topologies were then used as input trees for the third group of analyses (FREQPARS).

Two groups of PAUP analyses were performed. Any transformation series with three or more character states was polarized if possible, but unordered. Only *Etheostoma davisoni* was designated as an outgroup, because then the most parsimonious arrangement of all the data will determine whether *E. vitreum* is the sister group to one of the following: the subgenus *Ammocrypta*, any species or combination of species of *Ammocrypta*, the subgenus *Boleosoma*, a clade consisting of the subgenera *Boleosoma* and *Ammocrypta*, the species pair *E. nigrum*-*E. longimanum*, the species *E. nigrum*, or the species *E. longimanum*. This tests Simons' (1992) assertion that *E. vitreum* is the sister group to the subgenus *Ammocrypta*.

Allozyme data were coded using each locus as the transformation series and the allelic arrays as the character states. The order or cost of transformation from one character state to another was directed by step matrices (Mabee and Humphries, 1993). Two kinds of step-matrix files (available from the authors upon request) were constructed. In the first, allozyme transformation series were directed by a series of step matrices that contained only character states observed in the study taxa. This allows coding of all observed allelic combinations; hypothetical ancestral character states at interior nodes of resulting phylogenetic trees were limited to character states observed in study taxa. The second step-matrix file contained a single, large step matrix of character states observed in extant taxa as well as all other possible allelic arrays. Because of restrictions on the number of characters allowed by PAUP, this approach can only accommodate five or fewer alleles (31 states) per locus. Therefore the original data matrix had to be reduced in size. This reduction was accomplished by deleting autapomorphic alleles from the data matrix for those taxa in which they occurred. For PAUP analyses the most parsimonious and several near most parsimonious topologies were saved for FREQPARS analysis.

Two groups of FREQPARS analyses were performed. The first included only the allozyme frequencies. The second included both allozyme frequencies and morphological characters. Morphological characters were treated as if they were fixed electromorphs for the taxa in which they occur. That is, the presence of a particular morphological character state in a particular taxon was considered homozygous and present in 100% of the population sampled. All topologies produced using BIOSYS-1 and PAUP analyses were tested using FREQPARS. Simons' (1992) tree was also tested in this fashion. Tree lengths resulting from these FREQPARS analyses were used as the final arbiter between competing hypotheses of phylogenetic relationships of the ingroup taxa. This is because these analyses include all available data pertaining to these species (i.e., both allozyme frequency data and morphological character states) and they provided greater discrimination among competing hypotheses.

RESULTS

Observed genotypic frequencies are provided in Table 1. All taxa were polymorphic for at least two loci, most taxa were polymorphic at 6–8 loci, and *Etheostoma beanii* was polymorphic at 10 loci.

Analysis of the allozyme frequency data using BIOSYS-1 produced two trees. These differed from each other only in the placement of *Etheostoma nigrum*. Rogers genetic distance, modified Rogers distance, Prevosti distance, and Edwards "E" distance methods all resulted in a topology wherein *E. nigrum* was the sister taxon to the subgenus *Ammocrypta* (Fig. 1). The Cavalli-Sforza and Edwards chord distance and Cavalli-Sforza and Edwards arc distance methods resulted in identical topologies, with *E. nigrum* the sister taxon to the species pair *E. longimanum-E. vitreum* (Fig. 2).

PAUP analysis of the allozyme data alone, using a series of step matrices that only allowed character states observed in the study taxa, produced two equally parsimonious trees (TL = 166 steps). Each contained a monophyletic subgenus *Ammocrypta* that was the sister group to *Etheostoma nigrum*, *E. longimanum*, and *E. vitreum*. In one tree (Fig. 3), *E. vitreum* was the sister group to the species pair *E. longimanum-E. nigrum*, and in the other (Fig. 4), *E. nigrum* was the sister group to the species pair *E. longimanum-E. vitreum*. PAUP analysis of the allozyme data alone, with the changes in transformation series directed by a single, large step matrix produced a single most parsimonious tree (TL = 132 steps) with a monophyletic subgenus *Ammocrypta* that was the sister group to a group consisting of *E. vitreum* and the species pair *E. longimanum-E. nigrum*. This tree has the same topology as that in Figure 3.

Total evidence PAUP analysis using a series of step matrices found a single most parsimonious tree (TL = 207) with a monophyletic subgenus *Ammocrypta* as the sister taxon to *Etheostoma vitreum*; these taxa formed the sister taxon to the species pair, *E. longimanum-E. nigrum* (Fig. 5). Total evidence PAUP analysis using the single, large step matrix resulted in two most parsimonious trees (TL = 174) with a monophyletic subgenus *Ammocrypta* that was the sister group to *E. vitreum*. In one tree (Fig. 6), *E. nigrum* is the sister group to a monophyletic group containing *E. longimanum*, *E. vitreum*, and the subgenus *Ammocrypta*. In the other tree (Fig. 5), the species pair *E. longimanum-E. nigrum* form a monophyletic sister group to *E. vitreum* and the subgenus *Ammocrypta*.

The most parsimonious tree examined in the FREQPARS analyses based on the allozyme data alone was 113.442 FREQPARS units long. This tree is isomorphic with one of the two most parsimonious trees based on the PAUP

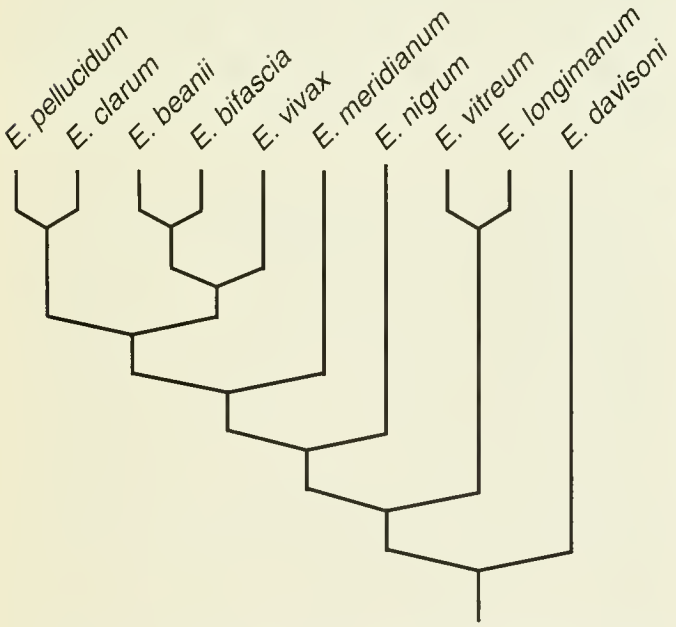

Fig. 1. Tree topology for ten species of *Etheostoma* resulting from BIOSYS-1 analysis of the allozyme data alone using Rogers genetic distance, modified Rogers genetic distance, Prevosti distance and Edwards "E" distance methods. This topology is 116.006 freqpars units when only the allozyme data are considered and 216.004 freqpars units for the total-evidence data.

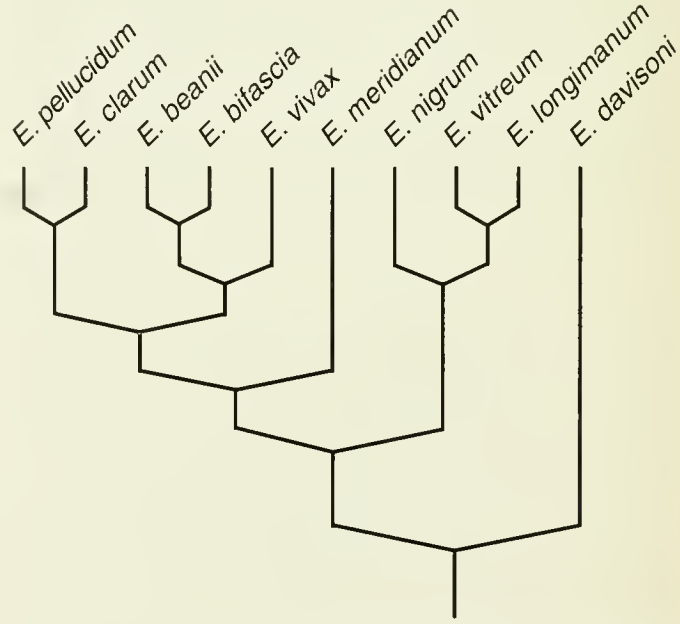

Fig. 2. Tree topology for ten species of *Etheostoma* resulting from BIOSYS-1 analysis of the allozyme data alone using Cavalli-Sforza and Edwards chord distance and the Cavalli-Sforza and Edwards arc distance methods. This topology is 115.606 freqpars units when only the allozyme data are considered and 211.604 freqpars units for the total-evidence data.

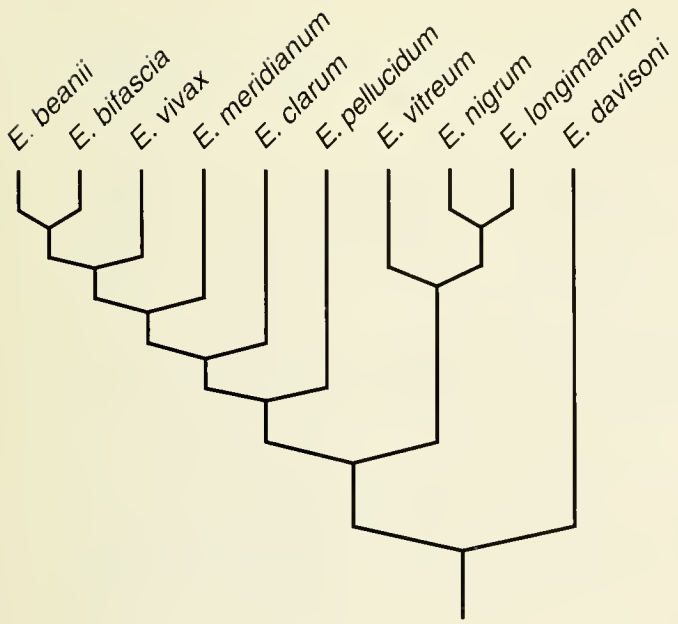

Fig. 3. The first of two most parsimonious trees (166 steps) for ten species of *Etheostoma* resulting from PAUP analyses of the allozyme data alone using a series of step matrices. This is also the single most parsimonious tree (132 steps) resulting from PAUP analyses of the allozyme data alone using a single large step matrix. This topology is 113.442 freqpars units when only the allozyme data are considered and 205.441 freqpars units for the total-evidence data.

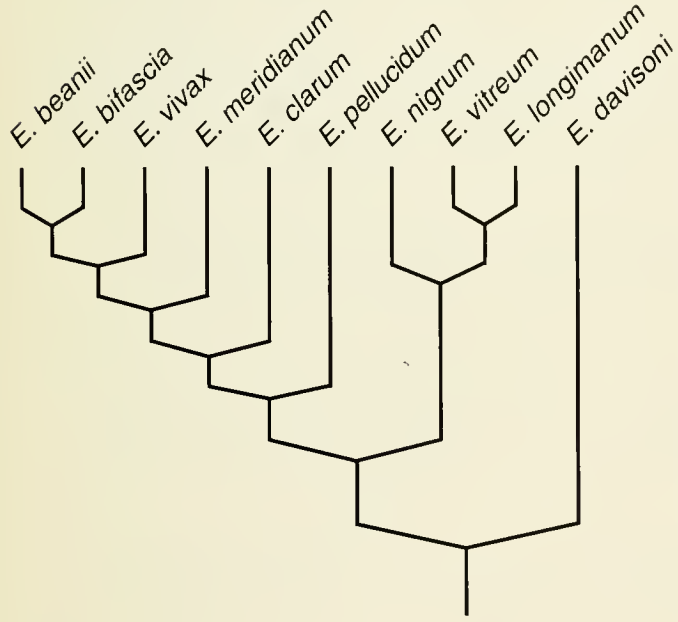

Fig. 4. The second of two most parsimonious trees (166 steps) for ten species of *Etheostoma* resulting from PAUP analyses of the allozyme data alone using a series of step matrices. This topology is 113.840 freqpars units when only the allozyme data are considered and 209.839 freqpars units for the total-evidence data.

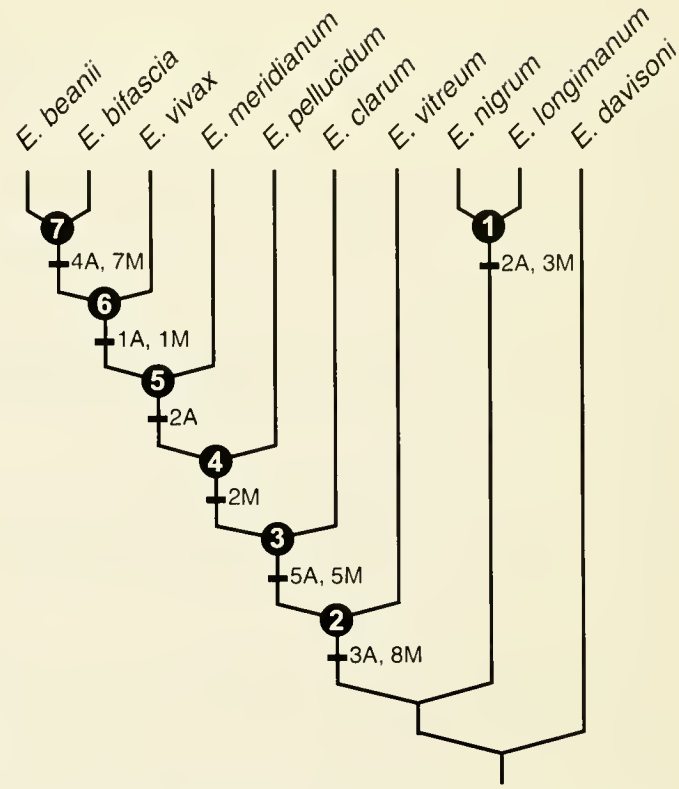

Fig. 5. The single most parsimonious tree (207 steps) for ten species of *Etheostoma* resulting from PAUP analyses of the total-evidence data using a series of step matrices. This is also the first of two most parsimonious trees (174 steps, the second is Fig. 6) resulting from PAUP analyses of the total-evidence data using a single large step matrix. This topology is 117.376 freqpars units when only the allozyme data are considered and 193.375 freqpars units for the total evidence.

Unambiguous character-state support (see text) resulting from PAUP analysis of the total-evidence data using a series of step matrices for each of the labeled nodes is as follows: **1.**—TS 1 (presence of c and loss of b), TS 13 (loss of d), TS 33:1 (curved ventral plate of the urohyal), TS 48:1 (thickened fin tips of breeding males), and TS 52:2 (female genital papillae flat and bilobed). **2.**—TS 15 (loss of a), TS 19 (presence of c and loss of b), TS 22 (loss of d or f, depending on which step matrix was used), TS 24:1 (posteriorly reclined ascending process of the premaxilla), TS 27:1 (palatine teeth absent), TS 29:1 (rectangular body of the quadrate), TS 30:1 (hyomandibular struts extremely reduced to absent), TS 34:1 (articular process for the interhyal on the posterior ceratohyal absent), TS 42:1 (vomerine teeth usually absent), TS 44:1 (remnant of the lateral-line canal on the supracleithrum absent), and TS 50:0 (tubercles on pelvic fins of breeding males present). **3.**—TS 7 (presence of b and loss of a), TS 11 (presence of d and loss of a), TS 13 (presence of e and loss of b), TS 14 (loss of c and presence of d or f, depending on which step matrix was used), TS 22 (presence of b and loss of e), TS 31:1 (descending process of hyomandibula short), TS 32:1 (hyomandibular spur present), TS 36:1 (notch in the anterior angle of the preopercle absent), TS 40:0 (mesethmoid thick and expanded anteriorly), and TS 43:1 (membrane bone on the lateral margin of the nasal reduced). **4.**—TS 35:1 (posterior margin of the preopercle serrate) and TS 39:1 (posterodorsal extension of the subopercle truncated). **5.**—TS 14 (presence of d and loss of e or f, depending on which step matrix was used) and TS 23 (presence of c and/or the loss of b, depending on which step matrix was used). **6.**—TS 22 (presence of a and/or loss of b, depending on which step-matrix was used) and TS 51:1 (tubercles on the anal fins of breeding males present). **7.**—TS 1 (presence of c and loss of b), TS 2 (presence of f and g and loss of d), TS 4 (presence of b and loss of c), TS 21 (presence of c), TS 26:1 (premaxillary socket of the maxilla U-shaped), TS 28:1 (notch posteroventral to the articular process of the quadrate deeply cut), TS 35:0 (posterior margin of the preopercle smooth), TS 38:1 (opercular strut greatly reduced), TS 45:1 (postcleithrum 2 absent), TS 46:1 (longitudinal struts on the anal proximal pterygiophores absent), and TS 49:1 (body squamation reduced laterally). **A.**—allozymic character states that support the node, **M.**—morphological character states that support the node.

Table 1. Genotypic distributions for 25 variable loci used in the quantitative phylogenetic analyses of the subgenus *Ammocrypta*.* The outgroup is *Etheostoma davisoni*.

Locus	Species of *Etheostoma*									
	davisoni	*longimanum*	*nigrum*	*vitreum*	*clarum*	*vivax*	*beanii*	*bifascia*	*meridianum*	*pellucidum*
M-Acon-A	aa (8)** ab (4) bb (3)	cc (15)	cc (15)	bb (1) bd (2) cc (6) cd (6)	bb (15)	aa (1) ab (2) bb (12)	cc (15)	cc (15)	bb (8)	bb (10)
S-Acon-A	aa (6) ab (2) bb (6) bd (1)	ee (15)	dd (15)	dd (15)	dd (15)	dd (12) de (3)	ff (9) fg (5) gg (1)	fg (1) gg (14)	bb (8)	cd (1) dd (9)
Ada-A	ee (15)	aa (15)	ac (1) cc (14)	bd (2) dd (13)	np (1) oo (2) op (8) pp (4)	mm(13) mo (1) oo (1)	gk (5) kk (7) kn (2) gm (1)	hh (3) hj (10) jj (2)	ff (1) fk (3) kk (4)	ii (7) il (3)
Ak-A	cc (14) cd (1)	cc (15)	cc (15)	cc (15)	cc (15)	cc (15)	bb (15)	bb (15)	cc (8)	ab (2) bb (8)
M-Aat-A	aa (12) ab (3)	aa (15)	bb (15)	aa (15)	aa (15)	aa (15)	aa (15)	aa (14) ab (1)	aa (8)	aa (10)
Ck-A	dd (15)	cc (15)	bb (15)	aa (15)	cc (15)	cc (15)	cc (15)	cc (15)	cc (6) cd (2)	cc (10)
Ck-B	aa (15)	aa (15)	aa (15)	aa (15)	bb (15)	bb (15)	bb (15)	bb (15)	bb (8)	bb (10)
Ck-C	bb (15)	cc (15)	cc (15)	cc (10) cd (4) dd (1)	cc (15)	cc (15)	ac (1) cc (14)	cc (15)	cc (8)	cc (10)
Gp-1	bb (15)	aa (15)	aa (15)	aa (15)	bb (15)	bb (15)	bb (15)	bb (15)	aa (8)	bb (10)
Gpi-A	cc (15)	dd (11) de (3) ee (1)	cc (15)	cc (15)	cc (15)	cc (14) cf (1)	cc (14) ce (1)	ac (1) cc (14)	bb (3) bc (2) cc (3)	bc (1) cc (9)
Gpi-B	ff (15)	aa (15)	ee (15)	aa (15)	bd (1) dd (14)	dd (15)	dd (14) df (1)	dd (15)	dd (6) df (2)	cd (1) dd (9)
Gapdh-A	cc (15)	cc (15)	cc (15)	aa (15)	cc (15)	bc (1) cc (14)	cc (15)	cc (15)	cc (8)	cc (10)
Glydh-A	bb (11) bd (4)	bb (14) cc (1)	bb (15)	bb (1) bd (4) cc (1) cd (1) dd (8)	de (1) ee (14)	de (3) ee (12)	de (1) ee (14)	de (2) ee (13)	aa (2) ae (4) de (2)	de (2) ee (8)

Table 1 continued

Locus	Species of *Etheostoma*									
	davisoni	*longimanum*	*nigrum*	*vitreum*	*clarum*	*vivax*	*beanii*	*bifascia*	*meridianum*	*pellucidum*
G3pdh-A	ee (15)	cc (15)	aa (15)	cc (15)	ff (15)	dd (15)	bd (1) dd (14)	dd (15)	dd (7) dg (1)	ff (10)
G3pdh-B	aa (15)	ab (6) bb (9)	bb (15)	bb (15)	bb (15)	bb (15)	bb (15)	bb (15)	bb (8)	bb (10)
S-lcdh-A	aa (15)	bb (15)	bb (15)	bb (15)	ab (1) bb (14)	bb (15)	bb (15)	bb (15)	bb (8)	bb (10)
Ldh-B	bb (15)	bb (15)	bb (15)	bb (15)	bb (15)	bb (15)	bb (15)	bb (15)	aa (4) ab (3) bb (1)	bb (10)
M-Mdh-A	cc (15)	cc (15)	ac (2) cc (6) cd (7)	bb (4) bc (7) cc (4)	cc (15)	cc (15)	cc (15)	cc (15)	cc (8)	cc (10)
S-Mdh-A	bb (15)	bb (15)	bb (15)	aa (15)	ab (1) bb (14)	ab (1) bb (14)	bb (15)	bb (15)	bb (8)	bb (10)
Mpi-A	bb (15)	cc (15)	bb (15)	cc (15)	cc (15)	cc (15)	cc (15)	cc (15)	aa (8)	cc (10)
Pgm-A	dd (15)	dd (15)	dd (15)	dd (11) fd (3) ff (1)	dd (15)	dd (15)	ab (1) bb (12) bd (2)	be (1) ce (1) ee (13)	dd (8)	dd (10)
Pgdh-A	aa (15)	aa (15)	bb (15)	aa (15)	dd (1) df (8) ff (6)	ee (15)	ce (1) ee (14)	ce (1) ee (14)	ee (8)	ee (10)
Pep-B	ee (13) ef (1) ff (1)	ee (14) ef (1)	dd (15)	ee (15)	bb (14) bc (1)	aa (15)	aa (15)	aa (12) ab (3)	bb (5) bc (3)	bb (10)
Tpi-A	bb (15)	ab (1) bb (14)	aa (15)	ab (5) bb (10)	bc (1) cc (14)	cc (15)	bc (1) cc (14)	cc (15)	cc (8)	bb (10)
Tpi-B	aa (15)	aa (15)	aa (15)	aa (15)	aa (15)	aa (14) ab (1)	aa (15)	aa (15)	aa (8)	aa (10)

*M-lcdh-A and Ldh-A were monomorphic.
**Letters refer to alleles; numbers of individuals scored are in parentheses.

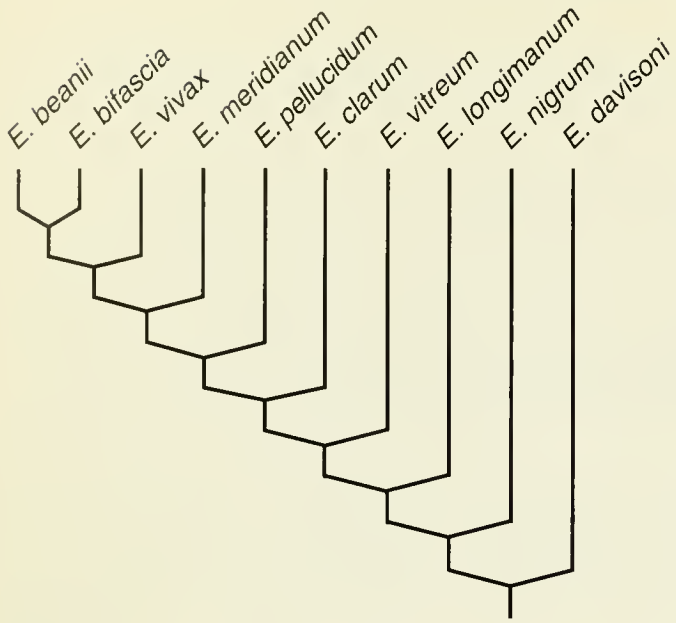

Fig. 6. The second of two most parsimonious trees (174 steps, the first is Fig. 4) for ten species of *Etheostoma* resulting from PAUP analyses of the total-evidence data using a single large step matrix. This topology is 115.974 freqpars units when only the allozyme data is used and 193.973 freqpars units for the total-evidence data.

analysis of the allozyme data alone when directed by a series of step matrices and to the single most parsimonious tree from the PAUP analysis of the allozyme data alone when directed by a single, large step matrix (Fig. 3). For allozyme data alone, the tree presented by Simons (1992; TL = 121.208) was considerably longer than the most parsimonious FREQPARS trees, as were the two trees found by BIOSYS-1 (TL = 115.606 and TL = 116.006).

The most parsimonious tree examined in all the FREQPARS analyses based on total evidence was 193.375 FREQPARS units. This tree is identical to the single most parsimonious tree produced by PAUP analysis of all data, when the allozyme data were directed by a series of step matrices, and to one of the two most parsimonious trees produced by PAUP analysis of all data when the allozyme data were directed by a single, large step matrix (Fig. 5).

Unambiguous character-state support (i.e., character states that support the monophyly of the group in both ACCTRAN and DELTRAN optimizations) for the total-evidence tree shown in Figure 5 is extensive. Five derived character states support the monophyly of the subgenus *Boleosoma*. Eleven unambiguous synapomorphies support *Etheostoma vitreum* as the sister group to the subgenus *Ammocrypta*, and 10 support monophyly of the subgenus *Ammocrypta*. Two unique, unambiguous character-state transformations each support three monophyletic clades: *E. pellucidum*, *E. meridianum*, *E. vivax*, *E. bifascia*, and *E. beanii*; *E. meridianum*, *E. vivax*, *E. bifascia*, and *E. beanii*; and *E. vivax*, *E. bifascia*, and *E. beanii*. The *E. bifascia-E. beanii* species pair is supported by 11 unambiguous synapomorphies.

DISCUSSION

The most parsimonious FREQPARS total-evidence tree (Fig. 5) differs from the morphology-only tree of Simons (1989, 1992) and the allozyme-only trees (Figs. 3, 4). This result strongly indicates that both types of data contributed to the shortest topology. For three branches the contribution of each type of data is essentially equal and therefore congruent. These branches subtend the following clades: subgenus *Boleosoma* (2 allozymic [A], 3 morphological [M]), subgenus *Ammocrypta* (5A, 5M), and *Etheostoma vivax*, *E. bifascia*, and *E. beanii* (1A, 1M). Two clades are best supported by the morphological data, but allozymic character states also support the relationships of *Ioa* and *Ammocrypta* (3A, 8M), and the species pair *E. beanii-E. bifascia* (4A, 7M). Two clades are supported by data that show complementarity (i.e., branches that would lack unambiguous support in analyses that have only one type of data). These include the clade consisting of *E. meridianum*, *E. vivax*, *E. bifascia*, and *E. beanii* (2A, 0M) and *Ammocrypta*, exclusive of *E. clarum* (0A, 2M).

The addition of allozymic data to the morphological data compiled by Simons (1992) results in a different set of relationships within *Ammocrypta* than those resulting from the analysis of morphological data alone. Figure 5 differs from Simons (1992) in the placement of *Etheostoma vivax*, *E. meridianum*, and *E. pellucidum*. In our total-evidence FREQPARS analysis, these three taxa are sequentially basal to the *E. beanii-E. bifascia* species pair not the monophyletic sister group to the species pair. Optimization of our total-evidence data onto the tree topology reported by Simons (1992) results in a FREQPARS tree length of 195.207 steps; this result is 1.832 FREQPARS steps longer than our most parsimonious total-evidence tree (Fig. 5). Moreover, relationships found in this study do not support either of the species groups proposed by Williams (1975) on the basis of external morphology. Optimization of our total-evidence data onto the tree implied by Williams (1975) was not possible, because he did not include all the taxa used in our study. We are therefore unable to provide a comparable FREQPARS length for our total-evidence data optimized onto Williams' (1975) set of relationships.

The analysis of both allozymic data and the morphological data together also results in a different set of relationships within *Ammocrypta* than those resulting from the analysis of the allozyme data alone. Within the subgenus

Ammocrypta, the allozyme-only analyses always placed *Etheostoma pellucidum* as the most basal member of the clade, whereas the total-evidence analyses placed *E. clarum* as the basal taxon. Overall, the most-parsimonious total-evidence topology for the subgenus *Ammocrypta* is more similar to the allozyme-only trees than the morphology-only trees.

The relationships of *Etheostoma vitreum* differed among the analyses. For the total-evidence trees (Figs. 5, 6) and the morphology-only trees (Simons, 1989, 1992) *E. vitreum* was always the sister taxon to the subgenus *Ammocrypta*. Simons' (1992) analysis identified seven characters that supported the monophyly of a clade composed of *E. vitreum* and the subgenus *Ammocrypta*, all of which were included in this study. Note that three allozymic character states also support this grouping. However, the allozyme-only trees had *E. vitreum* more closely related to members of the subgenus *Boleosoma* than to members of the subgenus *Ammocrypta*. Wood and Mayden (1997) found similar results in an allozyme analysis. Simons (1992) also identified two character states that potentially supported an *E. vitreum-Boleosoma* clade—attachment of eggs on rocks and darkened breeding coloration. Members of the subgenus *Boleosoma* attach their eggs to the undersurfaces of rocks above the substrate (Page, 1985) and *E. vitreum* attach their eggs to the vertical surfaces of rocks (Winn and Picciolo, 1960). Other darters bury their eggs in the sand and gravel substrate, clump their eggs under large rocks where the rock meets the gravel substrate, and deposit their eggs on vegetation and submerged debris such as leaves, twigs, and roots (Page, 1985). The preferred spawning location of most species of *Ammocrypta* is unknown, except for *E. pellucidum* that bury their eggs in a sand and gravel substrate (Johnston, 1989). The preference for attaching eggs to the undersurfaces of rocks is not unique to *Boleosoma*; this behavior is also true of members of the subgenus *Catonotus* (Page, 1985). Moreover, Simons (1992) also argued that attaching eggs to the undersurfaces of rocks was probably not the same character state as attaching eggs to the vertical surfaces of rocks. Breeding males of *Boleosoma* (Cole, 1967) and *E. vitreum* (Winn and Picciolo, 1960) darken in the breeding season and lack any other bright coloration. Although many breeding male darters exhibit brilliant hues of red, orange, yellow, green, and blue, many breeding males also have darkened bodies (e.g., members of the subgenera *Etheostoma, Vaillantia, Nothonotus*, and *Catonotus*) (Page, 1983). These two character states do not strongly unite *E. vitreum* and *Boleosoma* because in each case, there are other taxa that share the derived state. The differences between the topologies resulting from the use of different data sets, do however, indicate that there is some homoplasy concerning the relationships of these taxa. In spite of this, the most parsimonious hypothesis (Fig. 5) shows three allozymic character states (TS 15: loss of a, TS 19: presence of c and loss of b, and TS 22: loss of d or f depending on which step matrix was used) and eight morphological character states (TS 24:1, TS 27:1, TS 29:1, TS 30:1, TS 34:1, TS 42:1, TS 44:1, and TS 50:0) that support Simons' (1992) hypothesis that *E. vitreum* is the sister taxon to the subgenus *Ammocrypta*.

The subgenus *Boleosoma* was monophyletic in some analyses and not in others. The most parsimonious FREQPARS total-evidence tree (Fig. 5, 193.375 FREQPARS units long) provides the best-supported resolution of relationships of the *Boleosoma* group. This tree has a monophyletic *Boleosoma* with *Etheostoma nigrum* and *E. longimanum* as sister taxa. This was also the result when a single, large step matrix directed the analysis of the allozyme data and one of the two trees resulting from the analysis of the allozyme data as directed by a series of step matrices (Fig. 3). An alternative, slightly longer FREQPARS total-evidence tree (Fig. 6, 193.973 FREQPARS units long) has a paraphyletic *Boleosoma* with *E. longimanum* as the sister group to *Ammocrypta-Ioa* and *E. nigrum* basal to these taxa. Additionally, the second of the two trees resulting from the analysis of the allozyme data as directed by a series of step matrices (Fig. 4) has a paraphyletic *Boleosoma* with *E. nigrum* sister to the species pair *E. vitreum-E. longimanum*. This diversity of results suggests that the monophyly of the subgenus *Boleosoma* might be questionable. Simons (1992) identified three character states that supported the monophyly of the subgenus (presence of white knobs on the paired fins of breeding males, the absence of male breeding tubercles, and flattened, bilobed female genital papillae). Of these, he expressed doubt regarding the validity of the presence white knobs on the paired fins of breeding males, because similar knobs are present in other taxa of darters. He noted that removal of this character from his analyses resulted in two additional equally parsimonious trees, in one of which the node supporting the monophyly of *Boleosoma* collapsed resulting in a paraphyletic *Boleosoma*. Our most parsimonious total-evidence FREQPARS tree (Fig. 5) showed a monophyletic *Boleosoma* supported by two allozymic character states (TS 1: presence of c and loss of b, and TS 13: loss of d) and three morphological character states (TS 33:1, TS 48:1, and TS 52:2), but the variation in trees suggests instability in this region of the tree.

Wiley and Mayden (1985) analyzed the pattern of speciation in several groups of aquatic vertebrates distributed along the northern Gulf Coastal Plain. They found that many groups had pairs of sister taxa with one species occurring in the Mississippi River and drainages to the west and with the other species occurring in drainages to the east of the Mississippi River. They suggested that this pattern resulted from a "vicariance event loosely associated

with the Mississippi River" (p. 605). This explanation requires that the taxa in question are sister taxa and it was hypothesized to be true for *Etheostoma clarum* and the species pair, *E. bifascia-E. beanii*. In the present study, these taxa do not form a monophyletic group (Fig. 5), and therefore this hypothesis is no longer valid. Wiley and Mayden (1985) also found that many groups had one sister species occurring in the Mobile Bay Basin and drainages to the west and the other sister species occurring in drainages to the east of the Mobile Bay Basin. They suggested that the distribution of *E. meridianum* and *E. vivax-E. pellucidum* was the result of a vicariance event in this region. Simons' (1992) results suggested that this explanation was possible (because these were sister groups), but he thought that the distributions were not close enough to make this explanation acceptable. He suggested that the more northern distribution of *E. vivax* did not fit Wiley and Mayden's (1985) explanation. Our results do not support these hypotheses, because sister group relationships among these taxa were not demonstrated (Fig. 5). However, the sister-group relationship between *E. bifascia* and *E. beanii* is strongly supported. Their distributions (*E. bifascia* in Gulf Slope drainages east of the Mobile Bay Basin and *E. beanii* in the Mobile Bay Basin and Gulf Slope drainages to the west) remain an excellent example of a vicariant speciation event associated with the origin of the Mobile Bay Basin. The distributions of the rest of the subgenus *Ammocrypta* are best explained as resulting from dispersal and differentiation, not vicariant speciation.

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APPENDIX 1

Specimens Examined.

All material examined is deposited in the ichthyological collection of the Natural History Museum of The University of Kansas (KU). Vouchers for the electrophoretic analysis include the remains of actual specimens (number of specimens indicated in parentheses).

Etheostoma beanii.—Pearl River Drainage: MISSISSIPPI: Leake Co.: Pearl River, 2 miles S of Carthage on Mississippi Highway 35. KU 22868 (15).

Etheostoma bifascia.—Escambia River Drainage: FLORIDA: Escambia Co.: Pine Barren Creek at Florida highway 29, just S of Pine Barren. KU 22146 (15).

Etheostoma clarum.—White River Drainage: ARKANSAS: Lawrence Co.: Strawberry River at Arkansas highway 25, 2.5 miles NE of Strawberry. KU 23145 (15).

Etheostoma davisoni.—Choctawhatchee River Drainage: ALABAMA: Bullock Co.: Sec. 26, T12N, R24E, Spring Creek at Bullock County Road 14, 11 miles SE of Union Springs. KU 23141 (15).

Etheostoma longimanum.—James River Drainage: VIRGINIA: Roanoke Co.: Catawba Creek at Route 311 bridge, 0.5 miles SE Catawba. KU 23142 (15).

Etheostoma meridianum.—Tombigbee Drainage: MISSISSIPPI: Noxubee Co.: Hashuqua Creek at highway 490, 5.6 mi. S of Mashulaville. KU 23148 (1). Winston Co.: Noxubee River, 5 mi. S of Sturgis. KU 23149 (7).

Etheostoma nigrum.—Kansas River Drainage: KANSAS: Wabaunsee Co.: East branch of Mill Creek at mouth of Nehring Creek. KU 23143 (15).

Etheostoma pellucidum.—Wabash River Drainage: INDIANA: Fulton Co.: Tippecanoe River at Talma. KU 23150 (10).

Etheostoma vitreum.—Roanoke River Drainage: VIRGINIA: Franklin Co.: Blackwater River at Route 220 bridge, 5.8 airmiles SE of Boones Mill. KU 23144 (15).

Etheostoma vivax.—St. Francis River Drainage: ARKANSAS: Clay Co.: St. Francis River at the Arkansas-Missouri border on Arkansas highway 90, 8.75 miles E and 1.5 miles S of Rector. KU 23146 (15).

APPENDIX 2

Enzymes, International Union of Biochemistry Nomenclature Committee numbers, loci, tissue sources, and electrophoretic conditions for examination of the subgenus *Ammocrypta*.

Enzyme	IUBNC No.	Locus	Tissue source	Electrophoretic conditions*
Aconitate hydratase	4.2.1.3	M-Acon-A	Brain/Eye	B
		S-Acon-A	Liver	D
Adenosine deaminase	3.5.4.4	Ada-A	Muscle	A
Adenylate kinase	2.7.4.3	Ak-A	Brain/Eye	E
Aspartate amino-transferase	2.6.1.1	M-Aat-A	Liver	D
Creatine kinase	2.7.3.2	Ck-A	Muscle	D
		Ck-B	Brain/Eye	E
		Ck-C	Brain/Eye	E
General protein	Nonspecific	Gp-1	Muscle	D
Glucose-6-phosphate isomerase	5.3.1.9	Gpi-A	Muscle	A
		Gpi-B	Muscle	A
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	Gapdh-A	Muscle	C
Glycerate dehydrogenase	1.1.1.29	Glydh-A	Liver	C
Glycerol-3-phosphate dehydrogenase	1.1.1.8	G3pdh-A	Muscle	C
		G3pdh-B	Liver	C
Isocitrate dehydrogenase	1.1.1.42	M-Icdh-A	Brain/Eye	B
		S-Icdh-A	Brain/Eye	B
L-Lactate dehydrogenase	1.1.1.27	Ldh-A	Liver	C
		Ldh-B	Liver	C
Malate dehydrogenase (NAD+-dependent)	1.1.1.37	M-Mdh-A	Brain/Eye	B
		S-Mdh-A	Brain/Eye	B
Mannose-6-phosphate isomerase	5.3.1.8	Mpi-A	Muscle	C
Phosphoglucomutase	5.4.2.2	Pgm-A	Muscle	A
Phosphogluconate dehydrogenase	1.1.1.44	Pgdh-A	Brain/Eye	B
Tripeptidase	3.4.11.4	Pep-B	Liver	D
Triose phosphate isomerase	5.3.1.1	Tpi-A	Brain/Eye	B
		Tpi-B	Brain/Eye	B

*A: Tris-citrate I-NADP pH 7.0 (a discontinuous buffer system using 0.22 M TRIS and 0.09 M citric acid for the electrode and 0.01 M TRIS and 0.003 M citric acid for the gel), 5.88 V/cm 8 h; B: Tris-citrate II pH 8.0 (Selander et al., 1971), 5.59 V/cm 8 h; C: Tris-citrate II-NAD pH 8.0, 5.59 V/cm 8 h; D: Histidine-citrate pH 7.0 (Fildes and Harris, 1966), 5.59 V/cm 8 h; E: Histidine-citrate-NADP pH 7.0, 5.59 V/cm 8 h.

APPENDIX 3

Character matrix used in the qualitative PAUP analyses for members of the subgenus *Ammocrypta*. The outgroup is *Etheostoma davisoni*. Allelic codes correspond to those in Table 3. Loci having more than one allele are in parentheses.

Character	Species of *Etheostoma*									
	davisoni	*longimanum*	*nigrum*	*vitreum*	*clarum*	*vivax*	*beanii*	*bifascia*	*meridianum*	*pellucidum*
1. M-Acon-A	(ab)	c	c	(bcd)	b	(ab)	c	c	b	b
2. S-Acon-A	(abd)	e	d	d	d	(de)	(fg)	(fg)	b	(cd)
3. Ada-A	e	a	(ac)	(bd)	(nop)	(mo)	(gknm)	(hj)	(fk)	(il)
4. Ak-A	(cd)	c	c	c	c	c	b	b	c	(ab)
5. M-Aat-A	(ab)	a	b	a	a	a	a	(ab)	a	a
6. Ck-A	d	c	b	a	c	c	c	c	(cd)	c
7. Ck-B	a	a	a	a	b	b	b	b	b	b
8. Ck-C	b	c	c	(cd)	c	c	(ac)	c	c	c
9. Gp-1	b	a	a	a	b	b	b	b	a	b
10. Gpi-A	c	(de)	c	c	c	(cf)	(ce)	(ac)	(bc)	(bc)
11. Gpi-B	f	a	e	a	(bd)	d	(df)	d	(df)	(cd)
12. Gapdh-A	c	c	c	a	c	(bc)	c	c	c	c
13. Glydh-A	(bd)	(bc)	b	(bcd)	(de)	(de)	(de)	(de)	(ade)	(de)
14. G3pdh-A	e	c	a	c	f	d	(bd)	d	(dg)	f
15. G3pdh-B	a	(ab)	b	b	b	b	b	b	b	b
16. S-Icdh-A	a	b	b	b	(ab)	b	b	b	b	b
17. M-Mdh-A	c	c	(acd)	(bc)	c	c	c	c	c	c
18. S-Mdh-A	b	b	b	a	(ab)	(ab)	b	b	b	b
19. Mpi-A	b	c	b	c	c	c	c	c	a	c
20. Pgm-A	d	d	d	(df)	d	d	(abd)	(bce)	d	d
21. Pgdh-A	a	a	b	a	(df)	e	(ce)	(ce)	e	e
22. Pep-B	(ef)	(ef)	d	e	(bc)	a	a	(ab)	(bc)	b
23. Tpi-A	b	(ab)	a	(ab)	(bc)	c	(bc)	c	c	b
24. Ascend. process premax.	0	0	0	1	1	1	1	1	1	1
25. Max. process premax.	0	1	1	1	1	1	1	1	1	1
26. Premax. socket of max.	0	0	0	0	0	0	1	1	0	0
27. Palatine teeth	0	0	1	1	1	1	1	1	1	1
28. Notch of quadrate	0	0	0	0	0	0	1	1	0	0
29. Body of quadrate	0	0	0	1	1	1	1	1	1	1
30. Hyomandibular struts	0	0	0	1	1	1	1	1	1	1
31. Decend. process hyoman.	0	0	0	0	1	1	1	1	1	1
32. Hyomandibular spur	0	0	0	0	1	1	1	1	1	1
33. Ventral plate of urohyal	0	1	1	1	0	0	0	0	0	0
34. Art. process of post. cerat.	0	0	0	1	1	1	1	1	1	1
35. Margin of preopercle	0	0	0	0	0	1	0	0	1	1
36. Notch of preopercle	0	0	0	0	1	1	1	1	1	1
37. Opercular spine	0	0	0	0	0	1	1	1	1	1
38. Opercular strut	0	0	0	0	0	0	1	1	0	0
39. Extension of subopercle	0	0	0	0	0	1	1	1	1	1
40. Mesethmoid shape	1	1	1	1	0	0	0	0	0	0
41. Maxillary ligament	0	1	2	2	0	0	0	0	0	0
42. Vomerine teeth	0	0	0	1	1	1	1	1	1	1
43. Membrane bone of nasal	0	0	0	0	1	1	1	1	1	1
44. Supracleith. canal remnant	0	0	0	1	1	1	1	1	1	1
45. Postcleithrum 2	0	0	0	0	0	0	1	1	0	0

Appendix 3 continued

Character	Species of *Etheostoma*									
	davisoni	*longimanum*	*nigrum*	*vitreum*	*clarum*	*vivax*	*beanii*	*bifascia*	*meridianum*	*pellucidum*
46. Anal pterygiophore struts	0	0	0	0	0	0	1	1	0	0
47. Process for *i. m.* muscle	1	0	0	0	0	0	1	0	0	0
48. Thick fin tips	0	1	1	0	0	0	0	0	0	0
49. Body scalation	0	0	0	0	1	0	1	1	0	0
50. Pelvic tuberculation	1	1	1	0	0	0	0	0	0	0
51. Anal tuberculation	0	0	0	0	1	1	1	1	0	0
52. Genital papilla shape	1	2	2	0	0	0	0	0	0	0